PHARMACEUTICAL BIOTECHNOLOGY

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1. **Pharmaceutical biotechnology**
2. **Introduction to Genetic Engineering or (rDNA) technology**

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1. Pharmaceutical biotechnology

- Since time of immemorial, living organisms have been used for producing useful compounds.
- Production of alcohol using yeast and fermented food.
- However, the real exploitation of living organism started with the discovery of penicillin around 1930.

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Pharmaceutical biotechnology.....

- Fermentation technology was first used in World War I to ferment corn starch and produce acetone for manufacturing explosives... acetone peroxide
  - with the help of *Clostridium acetobutylicum*
- In the 1950's, cholesterol was converted to cortisol and sex hormones by reactions such as microbial hydroxylation

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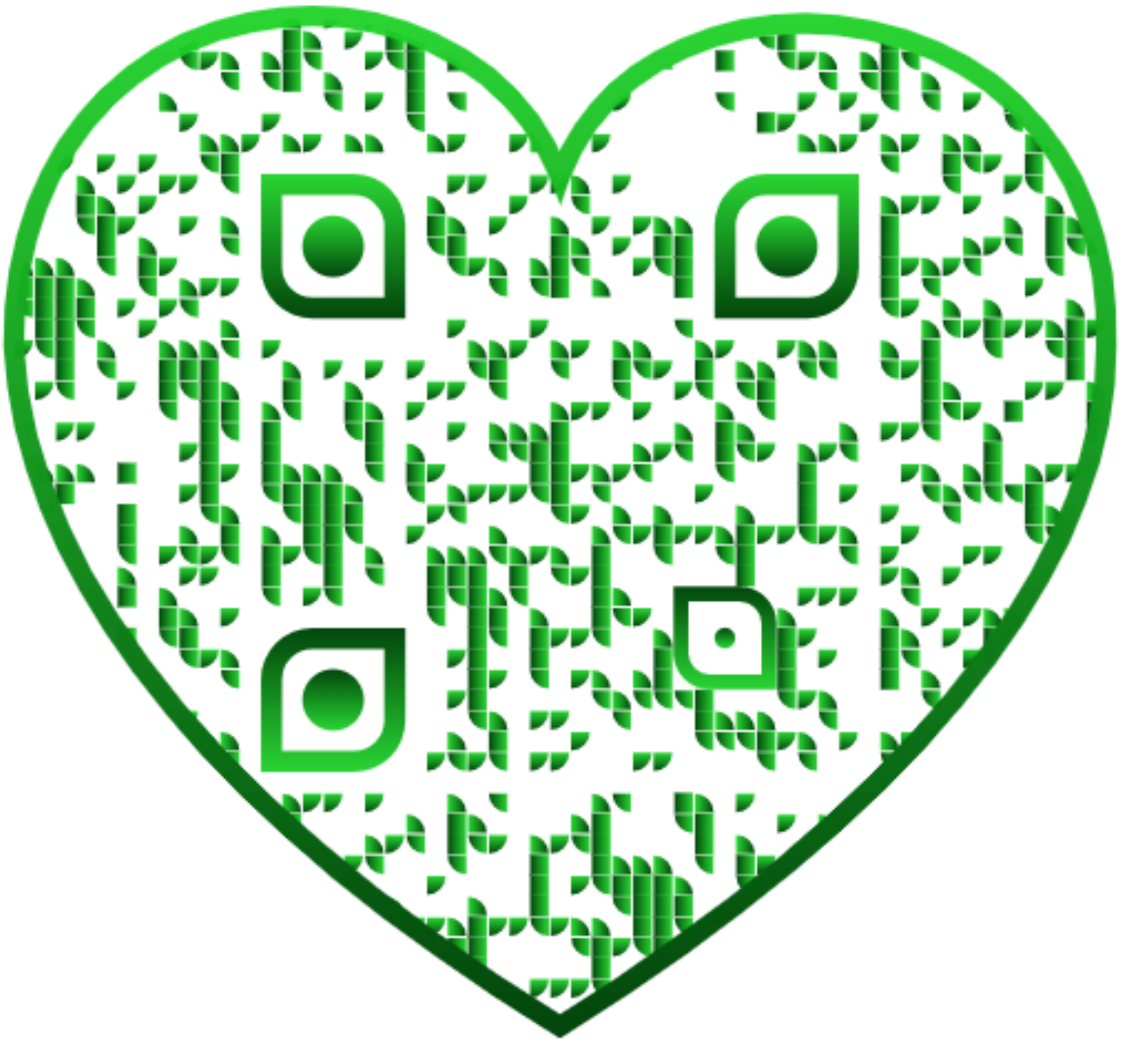
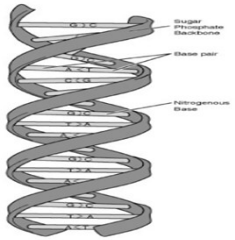


- 1953- Watson and Crick
  - **Determined the structure of DNA**
  - **DNA replication model -1953**
  - **DNA bases made up of purine & pyrimidine**
  - **Nobel Prize – 1962**



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Pharmaceutical biotechnology.....

- An enhanced understanding of protein structure, a detailed elucidation of cell replication and protein synthesis
- The isolation of DNA replication enzymes, including restriction enzymes and polymerases
  - Led to the rapid development of rDNA technology, permitting cloning and expression of proteins and peptides.

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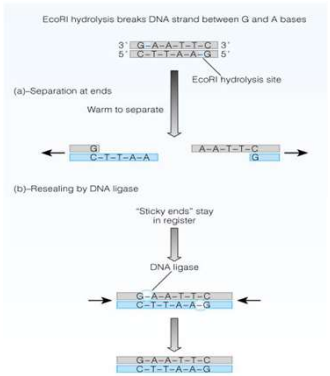
First Recombinant experiments

- 1971 scientists manipulated DNA and placed it into bacteria
- 1972 scientists joined two DNA molecules from different sources using
- the **endonuclease EcoRI** (to cut) and DNA **ligase** (to reseal)

rDNA



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Pharmaceutical biotechnology……..

- In 1975, scientists developed monoclonal antibody technology.
- Used as a tool to characterize and purify proteins that would selectively bind to respective antibodies with high specificity.
- These tools for preparation and characterization of recombinant products are essential for developing macromolecules into therapeutic products.

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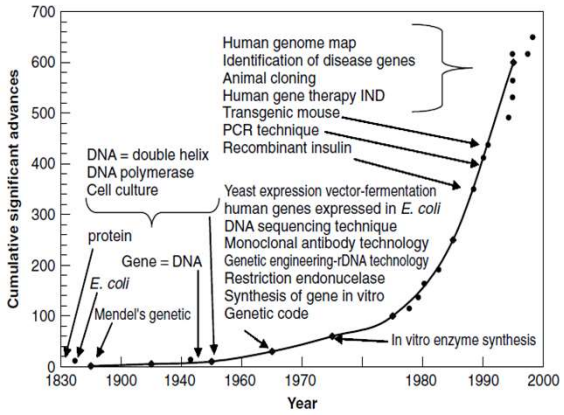

Figure 1.1. Milestones in pharmaceutical biotechnology.

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Pharmaceutical biotechnology……..

- the aggregate they have led to the development of dozens of pharmaceutical products that could not have been realized without the availability of these technologies.
- definition of pharmaceutical biotechnology include all materials made or derived from natural materials that can be used as drugs for human or animal medicine.

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Pharmaceutical biotechnology……..

Definition:

- **use of living organisms such as microorganisms to create new pharmaceutical products, or safer & more effective versions of conventionally produced pharmaceuticals, more cost-effectively.**
  - Use of biological systems to produce drug substances
  - Exploitation of modern technologies to produce drug substances from biological systems

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Biopharmaceuticals

- Class of therapeutic protein produced by modern biotechnological techniques, specifically via genetic engineering (in the case of monoclonal antibodies) or by hybridoma technology.

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- More recently, however, nucleic acids used for purposes of gene therapy and antisense technology have come to the fore and they too are generally referred to as 'biopharmaceuticals'.
- Biopharmaceutical : A protein or nucleic acid based pharmaceutical substance used for therapeutic or in vivo diagnostic purposes.

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Biotechnology medicines

- Such products encompass, for example, antibiotics extracted from fungi, therapeutic proteins extracted from native source material (e.g. insulin from pig pancreas) and products produced by genetic engineering (e.g. recombinant insulin).

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2. Genetic Engineering or (rDNA) technology

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Introduction

- Genes are sections of DNA on a chromosome.
- Each gene encodes for specific amino acid sequence to synthesize a protein

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Introduction to Genetic Engineering or (rDNA) technology

- the process of manipulating genes outside of a cell's/ organism's normal reproductive process.
  - It generally involves the isolation, manipulation and subsequent reintroduction of stretches of DNA into cells
- The collection of techniques for manipulating DNA and making recombinant molecules is known as rDNA technology /genetic engineering.
- *The **deliberate modification** of an organism's genetic information by directly changing its nucleic acid genome is called Genetic Engineering or recombinant DNA technology or gene cloning.*

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- 'rDNA technology' is a term used interchangeably with 'genetic engineering'.
- rDNA is a piece of DNA **artificially created *in vitro*** *which* contains DNA (natural or synthetic) obtained from two or more sources.

⇒usually undertaken in order to confer on the recipient cell the ability to produce a specific protein, such as a **biopharmaceutical**.

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rDNA …

- The fundamental techniques involved in working with rDNA involves
  - isolating or copying a gene
  - inserting the precise gene into a transmissible vector
  - transferring it to that host cell; and facilitating the transcription into mRNA and translation into proteins.

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Recombinant DNA technology tools

- DNA to be cloned
- Enzyme
  - **polymerases** -produce new strands of DNA.
  - **Endonucleasese** -They can cut DNA at specific locations
  - **Ligase** -It can attach segments of double stranded DNA

  -- **Reverse Transcriptase** -It synthesizes cDNA/cloned from mRNA.
- Vector/ Route
  - Plasmids, Cosmids etc
- Host
  - Bacteria, Yeast, etc

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Overview:

Isolate DNA

↓

Cut with restriction enzymes(cleavage)

↓

Ligate into cloning vector

↓

transform rDNA molecule into host cell

↓

Each transformed cell will divide many, many times to form a colony of millions of cells, each of which carries the rDNA molecule (DNA clone).

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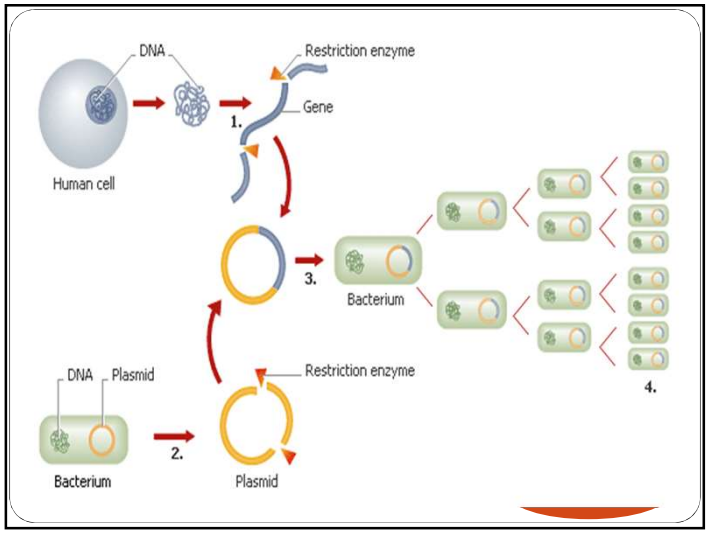

a) Isolating donor and plasmid DNA

I. Isolating donor DNA

⇒Cellular disruption => **solvent based extraction**

- Crude isolation of donor (foreign) DNA is accomplished by isolating cells ⟶ disrupting lipid membranes with detergent ⟶ destroying proteins with phenol or protease⟶ degrading RNAs with RNase ⟶leaving DNA at the end.

- Plants and some microorganisms, initial disruption of the cell wall may require application **of physical or other vigorous disruptive influences.**

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- The gentlest method of cell lysis usually involves incubation **with** cell-wall-degrading enzymes, and detergent
  - Following cellular disruption,
    - solvent-based extraction/precipitation.
      - E.g, shaking in the presence of **phenol** results in extraction of
      - Denatured proteins into the phenol phase / interphase,
      - Nucleic acids remaining in the aqueous phase.

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II. Isolating plasmid DNA

=> **Density gradient centrifugation** is the method employed for the isolation of bacterial plasmid.

- During this technique, a tube containing solution of Cesium Chloride and Ethidium Bromide- solvent is spun at high speed in centrifuge.
- This distributes the **Cesium Chloride** in a continuous concentration gradient, with the top segment containing the least concentration of Cesium Chloride and the bottom high concentration.
- Each DNA will form a separate band. The band containing plasmid can be collected and used for genetic engineering

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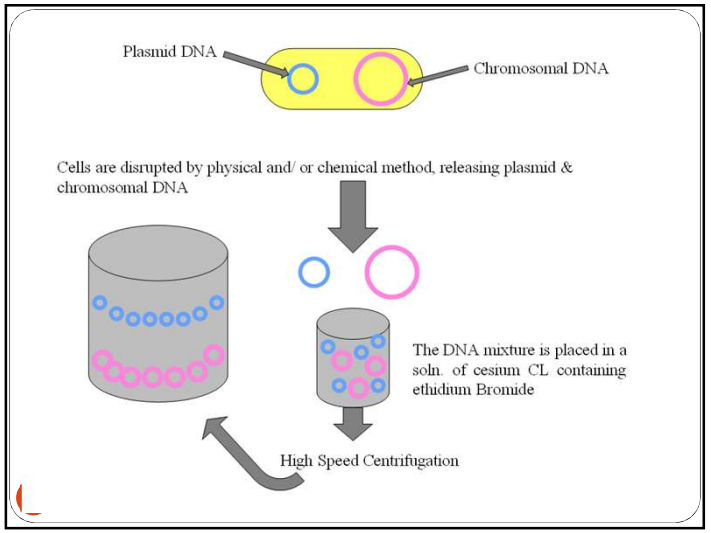
Plasmid DNA
Chromosomal DNA
Cells are disrupted by physical and/ or chemical method, releasing plasmid & chromosomal DNA
The DNA mixture is placed in a soln. of cesium CL containing ethidium Bromide
High Speed Centrifugation

B) Cutting of donor DNA and plasmid

- Both the plasmid DNA and donor DNA must be cleaved in such a way that the **sticky ended fragments are formed**
- DNA can be cut into large fragments by
  - mechanical shearing or Enzymes
- Restriction endonucleases
  - Are **bacterial enzymes** that cleave internal phosphodiester bonds of a DNA molecule.
  - **Are the scissors of molecular genetics**
  - More than **500 restriction endonucleases** have been discovered, and these react with more than 100 different cleavage sites.

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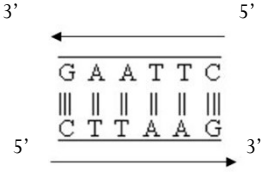
Restriction endonucleases…

- recognize a specific palindromic nucleotide sequence (**4-8 base pair**) on a double-stranded DNA molecule, called a restriction site, and
  - cleave the DNA at this recognition site in the DNA.
  - 3’ 5’



- Palindromic sequence in DNA is one in which the 5’ to 3’ base pair sequence is identical on both strands.

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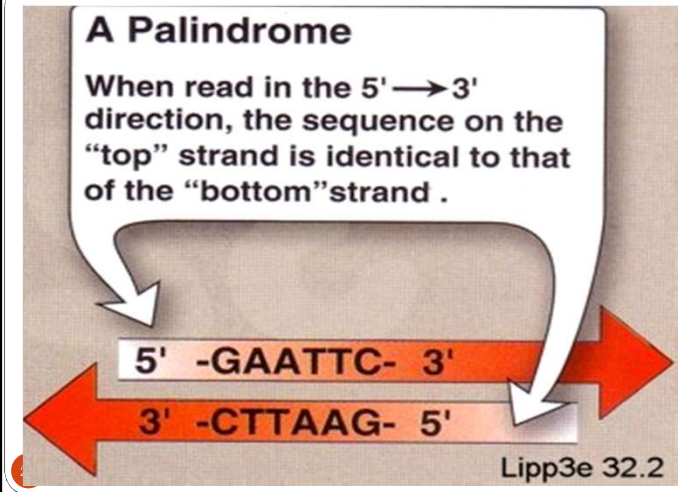
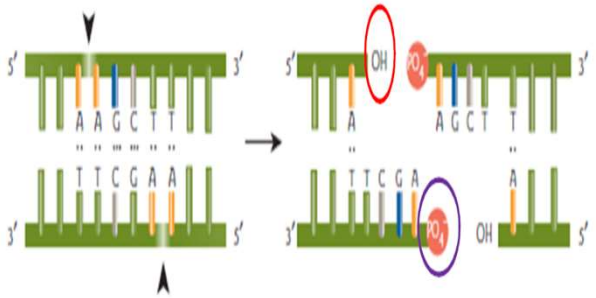

A Palindrome
When read in the 5' → 3' direction, the sequence on the "top" strand is identical to that of the "bottom" strand .
5' -GAATTC- 3'
3' -CTTAAG- 5'
Lipp3e 32.2

- The chemical reaction of the restriction endonuclease releases,
  - 3' end of one base as an alcohol and
  - 5' end as a monophosphate.



- Six base pair cutters are the most commonly used in molecular biology research.
- Restriction endonucleases **catalyze the hydrolysis of phosphodiester bonds** in palindromic DNA sequences
  - Formation of 5′-PO4− and 3′-OH termini

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C. Joining DNA

- **DNA ligase** is the glue of molecular genetics that holds the ends of the DNAs together.
- DNA ligase creates a **phosphodiester bond** between two DNA ends.
- This joining of linear DNA fragments together with covalent bonds is called **ligation**.

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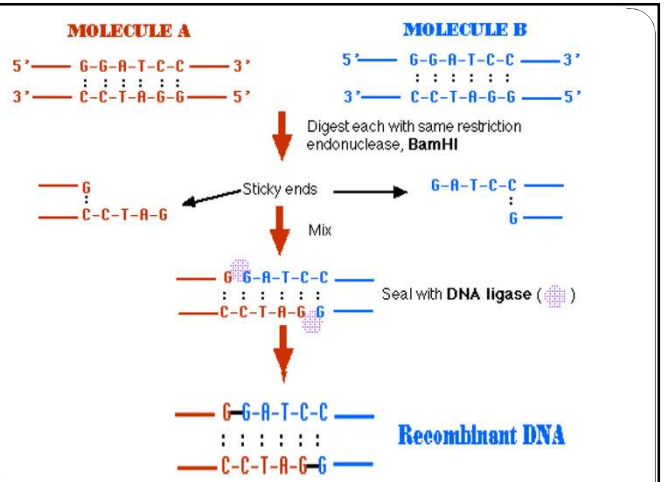

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D. Transforming recombinant plasmid and Amplifying the recombinant DNA

- To recover large amounts of the recombinant DNA molecule, it must be **amplified**.
- There are mainly two different ways that are used to amplify (clone) rDNA products:

I) *In vitro cloning: This is accomplished via Polymerase Chain* Reaction (PCR)

II) *In vivo cloning : using cellular prokaryotes (e.g E.coli)* , unicellular eukaryotes (e.g yeast, or mammalian tissue culture cells.

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D. Transforming…

- In vivo: transforming the recombinant DNA into a bacterial or other host strain

1) To incubate the cells (rDNA and the host) in a concentrated Ca salt solution
   - Make host membrane leaky=> so that the rDNA can easily enter the cell.
2) Using Electroporation
   - Drive rDNA into cells by a strong electric current
- Once in a cell, the recombinant DNA will be replicated. When the cell divides, the replicated recombinant molecules go to both daughter cells which themselves will divide later. Thus, the DNA is amplified.

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Invivo … Vector DNA

- **Vectors** are genetic elements that have been engineered and used for the insertion of genetic material into a cell.
- A DNA molecule originating from a virus, a plasmid or the cell of a higher organism into which another DNA fragment can be integrated without loss of the vector's (carrier's) capacity for self-replication.

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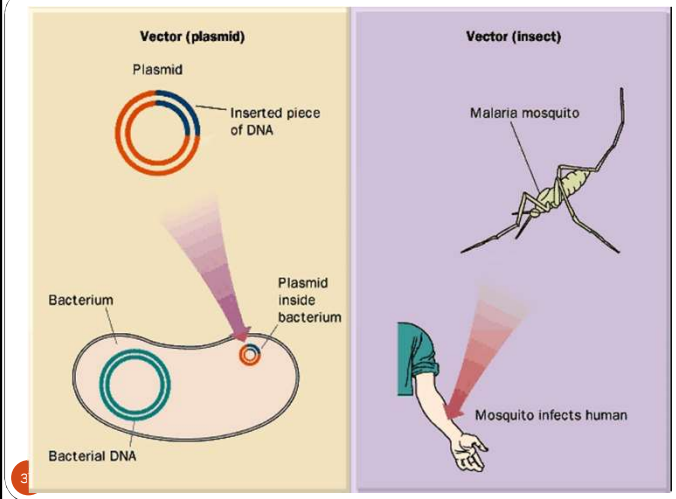

- Four important features of all cloning vectors are:

1. Can independently replicate themselves and the foreign DNA segments they carry

2. contain a number of unique restriction endonuclease cleavage sites

3. carry a selectable marker
   - Such as, antibiotic resistance genes
   - Their very purpose is to distinguish host cells that carry vectors from host cells that do not contain a vector

4. are relatively easy to recover from the host cell

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Invitro
PCR (Polymerase Chain Reaction)

It is a fast and inexpensive technique used to **amplify small and targeted segments of DNA to produce million of copies, sometimes called "molecular photocopying" of a specific gene fragment.**

- It is a technique used to study the molecular pathogenesis and diagnosis of a variety of acquired, inherited, viral and bacterial diseases.

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PCR Components

- A basic PCR technique requires certain components and reagents that include:

1. **Two primers (Forward & Reverse): short pieces of artificially prepared DNA that will target the gene fragment of interest in the entire genome.**

- **They are complementary to the 3' ends of each strand of the double stranded target gene i.e. DNA**

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PCR Components ...

2. Taq polymerase (DNA polymerase): It's an enzyme whose function is to extend the new DNA strand.

- ***Taq polymerase attaches*** *near the end of the primer and start adding nucleotides.*
- The DNA polymerase in our bodies breaks down at temperatures above 95 °C -- the temperature necessary to separate two complementary strands of DNA in a test tube.
- The DNA polymerase (Taq polymerase) that's used in PCR comes from a strain of bacteria called **Thermus aquaticus** that live in the hot springs.
- It can survive near boiling temperatures and works well at 72 °C.

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PCR Components...

3. Deoxynucleotide triphosphate (dNTP's):They are the building blocks from which the DNA polymerases synthesizes a new DNA strand.

- Taq polymerase grabs nucleotides that are floating in the liquid around it and attaches them to the end of a primer

4. Buffer solution: providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

5. MgCl2: acts as a **cofactor** for the polymerase enzyme. – aids enzymes activity.

6. Extracted DNA sample: containing the target region to be amplified

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PCR Steps:

PCR is a three-step process which is repeated in several cycles. The three steps are:

1. **Denaturation step:This step consists of heating the reaction to 90–95 °C.**

- **It causes** DNA separation **by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA. – cloned DNA**

2. Annealing step:The reaction temperature is lowered to 50–65 °C allowing hybridization of the primers **to the single-stranded DNA template.**

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PCR Steps...

3. Extension/Elongation step: At this step, the Taq polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction

- This process is repeated as many as 30 or 40 times, leading to more than one billion exact copies of the original DNA segment.
- The entire cycling process of PCR is automated and can be completed in just a few hours using a machine called a thermal cycler.

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PCR....

- Advantages:
  - Much faster than using vectors. Numb. Of copies=2^n-2n.. Where n is cycle
  - Only very small amount of target DNA is needed.

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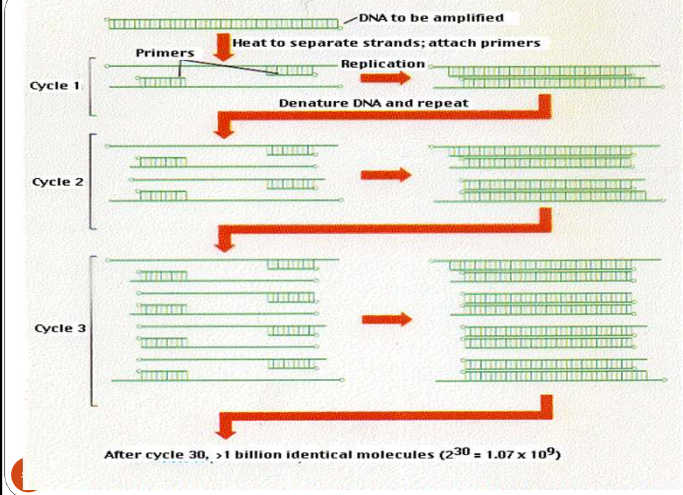

rDNA—invivo

- Most of the rDNA products approved by FDA are being produced using the following hosts:

Hosts for Recombinant Protein Production

S.No.	Hosts
1.	*Escherichia coli*
2.	*Bacillus subtilis*
3.	*Streptomyces*
4.	*Saccharomyces cerevisiae*
5.	*Pichia pastoris*
6.	*Aspergillus*
7.	Animal cells (CHO, SP20/NSO)
8.	Insect cells: Baculovirus system
9.	Transgenic Animals
10.	Transgenic Plants

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rDNA

- Currently, the most important expression systems used in the industry are the following :
  - Bacteria (*Escherichia coli [E. coli]*
  - Yeast (*Saccharomyces cerevisiae and Pichia pastoris]*
  - Mammalian cell (Chinese hamster ovary (CHO) cells)
  - Transgenic animals
- The two most important recombinant biomolecules, insulin and erythropoietin were produced using *E. coli and mammalian (CHO) cells respectively.*
- Most of the recombinant biomolecules are produced industrially using these two organism followed by yeast.

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rDNA

The choice of production systems depends on

1. The expression level
2. Quality of biomolecules, safety, manipulation of genetic system
3. The cost of production.
4. Requirement of some proteins to be modified, or glycosylated, in order to retain bioactivity, which can only be attained in certain expression systems.

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1. Prokaryotic

- Bacteria such as *Escherichia coli are widely used for the* expression of rDNA products.

Advantages

- high level of recombinant protein expression,
- rapid growth of cell and
- simple media requirement.

Disadvantage

- intracellular accumulation of heterologous proteins,
- the potential for product degradation due to trace of protease impurities and
- production of endotoxin.

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2. Eukaryotic

i. **Yeast**

- Yeast such as *Saccharomyces cerevisiae, Hansenulla polymorpha and Pichia pastoris are among the simplest eukaryotic organisms.*

Advantage:

- They grow relatively quickly and
- Are highly adaptable to large-scale production.
- These organisms do not produce endotoxin.
- They are capable of glycosylating proteins up to a certain extent like mammalian cells.

N.B: Yeast has been the most widely used fungus for production of proteins and vaccines (because of its easy growth and glycosylation capacity next to Chinese hamster ovary (CHO)).

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ii. Mammalian systems

- Example: Chinese hamster ovary (CHO) cell and Baby hamster kidney (BHK) cell
- They are the choice for production of human therapeutic proteins.

Advantage:

- Production of complex protein such as glycoprotein

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ii. Mammalian systems

Disadvantage:

- Mammalian cell culture is complex process, time consuming and
- cost of production of the products using these systems is high
- Slow growth and expensive nutrient media.

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- N.B: In spite of these disadvantages, in comparison to the microbial system, mammalian cells have been most extensively used for the production of glycoprotein and monoclonal antibodies.
- Expression system for glycoprotein
  - CHO cell >Yeast> Insect cell > Plant based

⇒ Glycosylation influences immunogenicity, ligand binding, stability, serum half life and to some extent efficacy of the recombinant molecules thus is very important in the case of hormones, blood factors and monoclonal antibody production.

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rDNA invivo…

3. Transgenic animals and plants

- Recent advances have been made in producing therapeutic proteins by using transgenic animals
  - Transgenic milk production is currently most feasible.

Advantages :

- are high expression levels and volume output,
- low capital investment, low operational costs

Disadvantage

- variation in product yield,
- contamination with agrochemical and fertilizer,
- impact of pest and disease, and

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rDNA advantages

- Recombinant DNA technology has had a **fourfold** positive impact upon the production of pharmaceutically important proteins:

1. It overcomes the problem of source availability.
   - Many proteins of therapeutic potential are produced naturally in the body in minute quantities.
- Examples: interferons, interleukins and colony-stimulating factors.
  - their direct extraction is impractical.
- Recombinant production allows the manufacture of any protein in whatever quantity it is required.

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rDNA advantages…

2. It overcomes problems of product safety

- Direct extraction of product from some native biological sources has, in the past, led to the unwitting transmission of disease.
- Examples include the transmission of blood-borne pathogens such as
  - hepatitis B and C and HIV via infected blood products and

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rDNA advantages…

3. It provides an alternative to direct extraction from inappropriate/dangerous source material.

- A number of therapeutic proteins have traditionally been extracted from human urine.
  - Follicle stimulating hormone (FSH) -urine of postmenopausal women
  - Human chorionic gonadotrophin (hCG)-the urine of pregnant women

4. It facilitates the generation of engineered therapeutic proteins displaying some clinical advantage over the native protein product.

- site-directed mutagenesis facilitate the logical introduction of predefined changes in a protein's amino acid sequence.

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APPLICATION OF GENETIC ENGINEERING

Broadly speaking genetic engineering plays its major role in

- production of drugs and biochemicals
- Genetically modified organisms
- Analysis of genetic diseases
- Correction of genetic defects

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APPLICATION OF GENETIC ENGINEERING…

Produce proteins to treat diseases and abnormalities.

- Insulin…….diabetes
- Growth hormone…….dwarfism
- Clotting factors……hemophilia
- TPA (clot buster)…….heart attacks
- Sex hormones……infertility
- Interferon……hepatitis C
- Interleukin-2……kidney cancer

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APPLICATION OF GENETIC ENGINEERING...

Genetically modified organisms

- **Transgenic Organism**
  -Has one or more genes from another species
- **Transgenic sheep**
  - Have human gene
  - Produce human proteins to treat disease
- **Microinjection**

  Fertilized sheep egg is injected with a human gene
  - AAT = alpha-1-antitrypsin to treat inherited emphysema

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APPLICATION OF GENETIC ENGINEERING...

- **Agricultural Application:**
- In agriculture, gene for herbicide resistant has been transplanted from bacteria into tobacco plants, demonstrating that plants can be better tolerates the herbicides.
- In 1988 researcher at Washington University introduce genes from a pathogenic virus into tomato plant cells and demonstrated that the cells would produce viral proteins at their surface and can block viral attack

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APPLICATION OF GENETIC ENGINEERING...

- **Increasing Variation**
- The ultimate source of genetic variation comes from mutations.
- Breeders can increase variation by inducing mutations.
- Useful for producing new kinds of bacteria
  - Ex. Oil digesting bacteria
    - Useful in cleaning up oil spills
    - Useful in producing new kinds of plants

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